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Published in:
The Journal of Biological Chemistry

DOI:
[10.1074/jbc.M211055200](https://doi.org/10.1074/jbc.M211055200)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2003

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Steen, A., Buist, G., Leenhouts, KJ., El Khattabi, M., Grijpstra, F., Zomer, AL., Venema, G., Kuipers, OP., Kok, J., & Leenhouts, K. J. (2003). Cell wall attachment of a widely distributed peptidoglycan binding domain is hindered by cell wall constituents. *The Journal of Biological Chemistry*, 278(26), 23874-23881. <https://doi.org/10.1074/jbc.M211055200>

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Cell Wall Attachment of a Widely Distributed Peptidoglycan Binding Domain Is Hindered by Cell Wall Constituents*

Received for publication, October 29, 2002, and in revised form, April 8, 2003
Published, JBC Papers in Press, April 8, 2003, DOI 10.1074/jbc.M211055200

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The C-terminal region (cA) of the major autolysin AcmA of *Lactococcus lactis* contains three highly similar repeated regions of 45 amino acid residues (LysM domains), which are separated by nonhomologous sequences. The cA domain could be deleted without destroying the cell wall-hydrolyzing activity of the enzyme *in vitro*. This AcmA derivative was capable neither of binding to lactococcal cells nor of lysing these cells while separation of the producer cells was incomplete. The cA domain and a chimeric protein consisting of cA fused to the C terminus of MSA2, a malaria parasite surface antigen, bound to lactococcal cells specifically via cA. The fusion protein also bound to many other Gram-positive bacteria. By chemical treatment of purified cell walls of *L. lactis* and *Bacillus subtilis*, peptidoglycan was identified as the cell wall component interacting with cA. Immunofluorescence studies showed that binding is on specific locations on the surface of *L. lactis*, *Enterococcus faecalis*, *Streptococcus thermophilus*, *B. subtilis*, *Lactobacillus sake*, and *Lactobacillus casei* cells. Based on these studies, we propose that LysM-type repeats bind to peptidoglycan and that binding is hindered by other cell wall constituents, resulting in localized binding of AcmA. Lipoteichoic acid is a candidate hindering component. For *L. lactis* SK110, it is shown that lipoteichoic acids are not uniformly distributed over the cell surface and are mainly present at sites where no MSA2cA binding is observed.

The major autolysin AcmA of *Lactococcus lactis* subsp. *cremoris* MG1363 is a peptidoglycan hydrolase that is required for cell separation and is responsible for cell lysis during stationary phase (1, 2). The 40.3-kDa secreted mature protein is subject to proteolytic degradation (3, 4) resulting in a number of activity bands in a zymogram of the supernatant of a lactococcal culture. Bands as small as that corresponding to a protein of 29 kDa were detected, all representing products of AcmA (2). From experimental data and homology studies, we inferred that AcmA consists of two domains: an active site domain and a C-terminal region containing three highly homologous re-

peats of 45 amino acids (cA), which might be involved in cell wall binding. Since the smallest active protein is 29 kDa, it was proposed that the protein undergoes C-terminal proteolytic breakdown (1, 2). Nearly all cell wall hydrolases seem to consist of a catalytic domain and usually, but not always, a domain containing a number of specific amino acid repeats (5, 6). Mur1 of *Streptococcus thermophilus* and Mur, the *N*-acetylmuramidase of *Leuconostoc citreum*, do not contain such repeats (7, 8). Peptidoglycan hydrolyzing activity of Mur could be detected *in vitro*, but Mur on its own was not able to complement an *acmA* mutation in *L. lactis*. However, expression of a Mur-cA fusion protein was able to play the role of AcmA in cell separation after cell division in *L. lactis acmA* (8).

Cell wall hydrolases of various bacteria and bacteriophages contain repeats similar to those present in AcmA. These repeats are also called LysM (lysine motif) domains, since they were originally identified in bacterial lysins (9). The presence of the LysM domains is not limited to bacterial proteins. They are also present in a number of eukaryotic proteins, whereas they are lacking in archaeal proteins (10).

A cell wall binding function has been postulated for a number of proteins containing LysM domains (5, 9, 11, 12). Partially purified muramidase-2 of *Enterococcus hirae*, a protein similar to AcmA and containing six LysM domains, binds to peptidoglycan fragments of the same strain (13). The p60 protein of *Listeria monocytogenes* contains two LysM domains and was shown to be associated with the cell surface (14). The γ -D-glutamate-meso-diaminopimelate murepeptidases LytE and LytF of *Bacillus subtilis* have three and five repeats, respectively, in their N termini and are both cell wall-bound (15–18). However, which particular parts of these enzymes entailed the binding capacity has not been examined in any of these studies.

Some spore-specific proteins in *B. subtilis* also contain LysM domains. His tag fusions of the spore proteins YdhD, YkuD, and YkvP (containing one LysM domain) were produced during sporulation and could be detected in mature spores (19, 20). SafA (YrbA), a protein involved in spore assembly, localized to the outer rim of the cortex and is apparently targeted to the spore by its N-terminal LysM domain (21). When the signal sequence of β -lactamase was replaced by the two N-terminal LysM domains of the spore protein YaaH, β -lactamase assembled in spores. The authors speculated that the LysM domain functions as a kind of signal sequence involved in assembly on forespores (20).

The structure of one of the two LysM domains of the *Escherichia coli* lytic transglycosidase MltD has been resolved by NMR studies (10). The domain has a $\beta\alpha\alpha\beta$ structure. In the same study also a potential substrate-binding site could be identified. A loop, present between β -strand 1 and α -helix 1 lies at the end of a shallow groove on the surface of the domain. A

* This work was supported in part by Unilever Research Laboratorium (Vlaardingen, The Netherlands). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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TABLE I
Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant phenotype(s) or genotype(s)	Source or reference
<i>L. lactis</i> subsp. <i>cremoris</i>		
MG1363	Lac ⁻ ; PrtP ⁻ ; Plasmid-free derivative of NCDO712	Ref. 45
MG1363 <i>acmA</i> Δ1	Derivative of MG1363 carrying a 701-bp <i>SacI</i> / <i>SpeI</i> deletion in <i>acmA</i>	Ref. 2
NZ9000	MG1363 <i>pepN::nisRK</i>	Ref. 32
NZ9700	Nisin-producing transconjugant containing the nisin-sucrose transposon Tn5276	Ref. 46
PA1001	Derivative of NZ9000 carrying a 701-bp <i>SacI</i> / <i>SpeI</i> deletion in <i>acmA</i> and a deletion in <i>htrA</i>	Laboratory collection
SK110	Phage resistant, contains galactosyl in LTA	Ref. 37
Other bacteria used		
<i>L. sake</i> NCBF2714	Wild type	Laboratory collection
<i>L. helveticus</i> ATCC 15009		ATCC
<i>L. casei</i> ATCC393	Type strain	ATCC
<i>E. faecalis</i>	Wild type	Laboratory collection
<i>S. thermophilus</i>	Wild type	Laboratory collection
<i>B. subtilis</i> 168	<i>trpC2</i>	Ref. 47
<i>E. coli</i> NM522	<i>supE</i> , <i>thi</i> , Δ(<i>lac-proAB</i>), Δ <i>hds5</i> (<i>r</i> ⁻ , <i>m</i> ⁻) [F ⁺ , <i>proAB</i> , <i>lacI</i> ^q <i>lacZ</i> ΔM15]	Stratagene (La Jolla, CA)
Plasmids		
pNG304	Cm ^r , pNZ8048 derivative containing the pre-pro sequence of <i>prtP</i> and <i>msa2</i> under control of P _{nisA}	Laboratory collection
pNG3041	Cm ^r , pNZ8048 derivative containing the pre-pro sequence of <i>prtP</i> and <i>msa2</i> and the C-terminal domain of <i>acmA</i> under control of P _{nisA}	Laboratory collection
pBluescript SK+	Ap ^r , general cloning vector	Stratagene
pGK13	Em ^r , Cm ^r , pWV01-based lactococcal cloning vector	Ref. 48
pGKAL1	Em ^r , Cm ^r , pGK13 containing <i>acmA</i> under control of its own promoter on a 1942-bp <i>SspI</i> / <i>Bam</i> HI insert	Ref. 2
pGKAL5	Em ^r , Cm ^r , pGKAL1 derivative expressing A ₁₋₂₁₈	This work
pDEL	Ap ^r , pBluescript SK+ with 348-bp <i>SacI</i> / <i>EcoRI</i> fragment of <i>acmA</i> obtained by PCR with primers ALA-4 and REPDEL-3	This work
pPA3	Cm ^r , pNZ8048 derivative containing the repeat domain of <i>acmA</i> under control of P _{nisA}	Laboratory collection

conserved aspartate or glutamate in this shallow groove could be involved in the interaction with the ligand (10).

In this paper, we describe that the LysM domains bind directly to peptidoglycan. The binding is not species-specific; the domain binds to many Gram-positive bacteria with different peptidoglycan structures. Using immunofluorescence microscopy, we show that the domain binds to specific loci on the cell surface. Specific chemical treatments of cells and cell walls indicate that a cell wall component, extractable with trichloroacetic acid, is responsible for hindering of AcmA binding, thereby causing this localized binding.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, Media, and Growth Conditions—The bacterial strains and plasmids used in this study are listed in Table I. *Lactococcus lactis* was grown at 30 °C in 2-fold diluted M17 broth (Difco) containing 0.5% glucose and 0.95% β-glycerolphosphate (G_{1/2}M17) or in undiluted M17 containing 0.5% glucose (GM17) when indicated. *L. lactis* subsp. *cremoris* SK110 was grown in M17 containing 0.5% lactose (LM17). For plasmid selection, chloramphenicol (Sigma) or erythromycin (Sigma) was added (5 μg/ml). *Lactobacillus* strains, *S. thermophilus*, and *E. faecalis* were grown in MRS medium (22) at 37 °C; *Lactobacillus sake* was grown at 30 °C. *E. coli* and *B. subtilis* were grown aerobically in TY broth (Difco) at 37 °C. For plasmid selection in *E. coli*, erythromycin (Sigma) or ampicillin (Sigma) was added at 100 μg/ml.

Chemicals and Enzymes—All chemicals used were of analytical grade and, unless indicated otherwise, obtained from Merck. Enzymes for molecular biology were purchased from Roche Applied Science and used according to the supplier's instructions. Purified peptidoglycan from *Staphylococcus aureus* was obtained from Fluka Chemie (Zwijndrecht, The Netherlands). Peptidoglycan from *Micrococcus luteus* and *Curtobacterium flaccumfaciens* were kind gifts of Prof. Dr. S. J. Foster (University of Sheffield).

Enzyme Assays and Optical Density Measurements—AcmA activity was visualized on G_{1/2}M17 agar plates containing 0.2% autoclaved lyophilized *Micrococcus lysodeikticus* ATCC 4698 cells (Sigma) as halos around *L. lactis* colonies after overnight growth at 30 °C. X-prolyl dipeptidyl aminopeptidase (PepX) was measured as described by Buist *et al.* (3). Absorbance was measured in a Novaspec II spectrophotometer

(Amersham Biosciences) at 600 nm. Autolysis of *L. lactis* was analyzed by following the absorbance at 600 nm of a 100-fold diluted overnight culture in fresh G_{1/2}M17 medium for 6 days at 30 °C.

DNA Manipulations and Transformations—Molecular cloning techniques were performed essentially as described by Sambrook *et al.* (23). Electroporation of *E. coli* and *L. lactis* was performed by using a gene pulser (Bio-Rad) as described by Zabarovsky *et al.* (24) and Leenhouts *et al.* (25), respectively. Miniprepations of plasmid DNA from *E. coli* and *L. lactis* were obtained by the alkaline lysis method as described by Sambrook *et al.* (23) and Seegers *et al.* (26), respectively. PCR products were purified using the High Pure PCR purification kit (Roche Applied Science).

Construction of AcmA Derivatives—A stop codon and *EcoRI* restriction enzyme site (italics and underlined in oligonucleotide REPDEL, respectively) were introduced in *acmA* at the end of the sequence specifying the active site domain. This was done by PCR using the primers REPDEL (5'-CGCGAATTCCCTTATGAAGAAGCTCCGTC) and ALA-4 (5'-CTTCAACAGACAAGTCC), annealing within the sequence encoding the signal peptide of AcmA and pGKAL1 as the template. The PCR product was digested with *SacI* and *EcoRI* and cloned into the corresponding sites of pBluescriptSK+, leading to pDEL. Subsequently, the 1187-bp *Pf*MI/*EcoRI* fragment of pGKAL1 (2) was replaced by the 76-bp *Pf*MI/*EcoRI* fragment of pDEL, resulting in pGKAL5, which was obtained in *L. lactis* MG1363*acmA*Δ1.

SDS-PAGE, Detection of AcmA Activity, Western Blotting, and Immunodetection—*L. lactis* cell and supernatant samples were prepared as described before (3). AcmA activity was detected by a zymogram staining technique using SDS-polyacrylamide (12.5 or 17.5%) gels containing 0.15% autoclaved, lyophilized *M. lysodeikticus* ATCC 4698 cells (Sigma) as described before (2). The standard low range and prestained low and high range SDS-PAGE molecular weight markers of Bio-Rad were used as references. SDS-polyacrylamide gels were stained with Coomassie Brilliant Blue (Bio-Rad).

Proteins were transferred from SDS-polyacrylamide (10%) gels to polyvinylidene difluoride membranes (Roche Applied Science) as described by Towbin *et al.* (27). MSA2 antigen was detected with 10,000-fold diluted rabbit polyclonal anti-MSA2 antiserum (28); AcmA was detected with 3000-fold diluted rabbit polyclonal anti-Acma antiserum (laboratory collection), using horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (Amersham Biosciences) and the ECL chemiluminescent detection system and protocol (Amersham Biosciences).

Cell Wall Isolation and Chemical Treatment—Cultures of *L. lactis* and *B. subtilis* (50 ml each) were pelleted, and cells resuspended in 5 ml of water were broken by vigorous shaking in the presence of glass beads (29). Nonbroken cells were removed by centrifugation (5 min, 2000 × g). The cell walls were isolated from the resulting supernatant by centrifugation (15 min, 20,800 × g). Cell walls were washed several times with water and lyophilized. Equal aliquots of cell walls (0.1 mg) were resuspended in 1 ml of water, 10% SDS, or 10% trichloroacetic acid and boiled for 15 min. Subsequently, the cell walls were washed, once with 1 ml of phosphate-buffered saline (PBS¹; 75 mM NaP_i, pH 7.3, 68 mM NaCl) and four times with 1 ml of water. *L. lactis* and *Lactobacillus casei* cells were treated with trichloroacetic acid by boiling 25 μl of cell culture in 1 ml of 10% trichloroacetic acid. *Lactobacillus helveticus* was treated with SDS by boiling 25 μl in 10% SDS. The cells were washed as described for the chemically treated cell walls.

Purification of PA3—*L. lactis* PA1001 containing plasmid pPA3 was grown overnight at 30 °C in GM17 supplemented with 5 μg/ml chloramphenicol. Subsequently, the cells were diluted 100-fold in 1 liter of fresh GM17 and induced with 1 ml of nisin-containing supernatant of an overnight culture of *L. lactis* NZ9700. The cells were cultivated for 24 h at 30 °C. Cells and supernatant were separated by centrifugation for 20 min at 10,000 rpm, after which the latter was filter-sterilized and concentrated with Vivaflow 200 (molecular mass cut-off of 10 kDa) to 20 ml and buffered with 80 ml of 25 mM sodium phosphate buffer, pH 5.8. To lower the salt concentration in the sample, 100 ml of H₂O were added. The sample was loaded on a 5-ml prepacked AKTA-prime SP-Sepharose column (Amersham Biosciences), which was equilibrated with 25 mM sodium phosphate buffer, pH 5.8. The column was washed with 25 mM sodium phosphate until the absorbance measured at 214 nm did not change anymore. Subsequently, bound proteins were eluted with a 200-ml NaCl gradient from 0 to 0.5 M. Fractions of 4 ml were collected. The fractions containing highly pure PA3 were collected and desalted using a high trap desalting column (Amersham Biosciences). The protein was kept in H₂O at −20 °C at concentrations ranging from 0.4 to 0.8 mg/ml.

Binding of Proteins to Lactococcal Cells, Cell Walls, and Purified Peptidoglycan—The cells of 2 ml of exponential phase cultures of MG1363acmAΔ1 were collected by centrifugation and gently resuspended in an equal volume of supernatant of similarly grown MG1363acmAΔ1 carrying either plasmid pGK13 (empty vector), pGKAL1 (expressing acmA), or pGKAL5 (expressing truncated acmA) and incubated at 30 °C for 20 min. Subsequently, the mixtures were centrifuged. Of the supernatants, 0.4 ml was dialyzed against three changes of 1 liter of demineralized water, after which they were lyophilized and dissolved in 0.2 ml of denaturation buffer (30). The cell pellets were washed with 2 ml of fresh medium, after which cell-free extracts were prepared in 1 ml of denaturation buffer.

Binding of the MSA2cA fusion protein was studied by growing *L. lactis* NZ9000 containing pNG304 or pNG3041 until an A₆₀₀ of 0.4 was reached. The cultures were induced with nisin (by adding one-one thousandth volume of the supernatant of the nisin producer *L. lactis* NZ9700) and incubated for 2 h. The cells of 1 ml of MG1363acmAΔ1 culture, 0.1 mg of cell walls, or 0.5 mg of peptidoglycan were resuspended in 1 ml of supernatant of either of the two induced cultures. Then the suspensions were spun down, and the pellets were washed with 1 ml of fresh M17 medium, resuspended in 100 μl of denaturation buffer (30), boiled for 5 min, and subjected to SDS-10% PAGE followed by Western blot analysis. The supernatant fractions, containing non-bound proteins, were concentrated 10 times using phenol and ether extraction (31). The concentrated supernatants were diluted with 1 volume of SDS sample buffer and subjected to SDS-10% PAGE.

To study the binding efficiency of MSA2cA to different peptidoglycan types, 50-μg samples of peptidoglycan isolated from *M. luteus* and *C. flaccumfaciens* were mixed with the protein. A dilution range of the protein was prepared by diluting the supernatant containing MSA2cA in supernatant of an *L. lactis* MG1363acmAΔ1 culture.

To study binding of PA3, 15 μg of purified PA3 was added to 70 μl of trichloroacetic acid-pretreated *L. lactis* cells (described above) in a total volume of 1 ml in 20 mM Tris-HCl, pH 8.0 (10¹⁰ cells/ml). The samples were incubated for 20 min on a blood cell suspension mixer at room temperature. Subsequently, the samples were spun down (3 min, 20,000 × g), and unbound proteins in the supernatant were precipitated with 5% trichloroacetic acid for 1 h on ice and subsequently centrifuged for 20 min at 20,000 × g. Precipitated proteins were washed with

acetone, air-dried, and resuspended in SDS-sample buffer. The cell pellets were washed with 1 ml of 20 mM Tris-HCl, pH 8.0, and resuspended in SDS-sample buffer. Samples were analyzed by SDS-PAGE and Coomassie staining.

Immunofluorescence Microscopy—Samples (25 μl) of bacterial cultures or the chemically treated bacterial cells were pelleted and washed once with 1 ml of water. The pellets were resuspended in *L. lactis* NZ9000 (pNG3041) culture supernatant containing MSA2cA and incubated for 5 min at room temperature. The cells were washed once with 1 ml of ½M17 and subsequently incubated for 10 min at room temperature in 100 μl of PBS containing 4% bovine serum albumin. Subsequently, the cells were resuspended in 100 μl of PBS containing anti-MSA2 antibody (diluted 1:400) with 2% bovine serum albumin for 1 h. After three washing steps with 1 ml of PBS containing 0.1% Tween 20 (Sigma) (PBST), the cells were incubated for 1 h in 2% bovine serum albumin in PBS, containing a 1:400 dilution of Oregon Green anti-rabbit antibody (Molecular Probes, Eugene, OR). Subsequently, the cells were washed three times with 1 ml of PBST and transferred to a poly-L-lysine-coated microscopic slide (Omnilabo, Breda, The Netherlands), which was air-dried at ambient temperature. Vectashield (Vector, Burlingame, CA) was added, a coverslip was mounted, and fluorescence was visualized with a Zeiss microscope (Carl Zeiss, Thornwood, CA) and an Axion Vision camera (Axion Technologies, Houston, TX).

Lectin Binding Studies—Fluorescein-labeled *Ricinus communis* agglutinin I lectin (RCA120) (Vector) was diluted to 10 μg/ml in lectin binding buffer (10 mM HEPES, pH 7.5, 0.15 M NaCl). *L. lactis* cells from 100 μl of overnight culture were spun down, and the pellet was resuspended in 100 μl of the diluted lectin. The suspension was incubated for 10 min on ice, and the cells were washed once in lectin binding buffer (100 μl) and resuspended in the same volume. Samples (5 μl) were spotted onto microscopic slides for fluorescence microscopy as described above.

RESULTS

AcmA Is a Modular Protein Containing an N-terminal Cell Wall-degrading Domain—We have previously analyzed the amino acid sequence of the *L. lactis* autolysin AcmA using Blast algorithms and PFAM and reported that mature AcmA probably consists of two domains (2). The N-terminal domain is predicted to contain the active site, whereas the C-terminal domain contains a putative cell wall binding domain. This cell wall binding domain consists of three homologous so-called LysM domains (9), separated by nonhomologous amino acid sequences (Fig. 1A). To investigate whether the N-terminal domain of AcmA indeed carries the active site, a deletion variant lacking the putative cell wall binding domain was created by introducing a stop codon downstream of the codon for Ser²¹⁸. The gene encoding truncated AcmA variant (A_{1–218}; see Fig. 1A) is expressed from the acmA promoter in the vector pGKAL5. The AcmA-negative strain *L. lactis* MG1363acmAΔ1 was transformed with pGKAL1, encoding wild type AcmA, or pGKAL5 and was plated on plates containing cell walls of *M. lysodeikticus*. Cells expressing acmA produced a clear halo around their colonies, whereas colonies of cells producing A_{1–218} did not. Cells containing pGKAL5 formed long chains, resulting in culture sedimentation, as has also been shown for the AcmA-negative strain *L. lactis* MG1363acmAΔ1 (2). Autolysis was substantially reduced upon deletion of the C-terminal domain. Whereas a 36.7% reduction of the absorbance at 600 nm was observed for *L. lactis* MG1363acmAΔ1 (pGKAL1) after 60 h of incubation at 30 °C, only 15.6% of A₆₀₀ reduction was observed for *L. lactis* MG1363acmAΔ1 expressing A_{1–218}. This reduction was similar to that of *L. lactis* MG1363acmAΔ1 (15.2%) (Table II). Only 0.3 arbitrary units of the intracellular peptidase PepX were present in the supernatant of the two AcmA-defective strains, whereas 19.8 arbitrary units were present in an equal volume of supernatant of the AcmA-producing culture *L. lactis* MG1363acmAΔ1 (pGKAL1).

Zymographic analysis of the cell fractions of overnight cultures of these three strains showed that AcmA and A_{1–218} are active (Fig. 1B). The truncated AcmA variant A_{1–218} is less

¹ The abbreviations used are: PBS, phosphate-buffered saline; LTA, lipoteichoic acid(s).

FIG. 1. A, schematic representation of pGKAL1 (expressing AcmA), pDEL, pGKAL5 (expressing A₁₋₂₁₈), pNG304 (expressing MSA2*), pNG3041 (expressing MSA2cA), and pPA3 (expressing PA3). SS (black), signal sequence of AcmA; SS (gray), signal sequence of Usp45 of *L. lactis*. Rx (dark gray), repeats; light gray, Thr-, Ser-, and Asn-rich intervening sequences (2); ppPrtP (black), signal and pro-sequence of the lactococcal proteinase PrtP (44). The AcmA active site domain is shown in white, and the *P. falciparum* antigen MSA2 is indicated by a striped bar. pDEL is a pBluescriptSK+ derivative containing the *SacI*/*EcoRI* fragment of the PCR products obtained with primers ALA-4 and REPDEL in the corresponding sites of pBluescriptSK+. The *Pf*MI/*EcoRI* fragment of pGKAL1 was replaced by the *Pf*MI/*EcoRI* fragment of pDEL, resulting in plasmid pGKAL5. B, left panel, Western analysis using AcmA-specific antibodies of AcmA antigen present in cell fractions of the equivalent of 1 ml of end-exponential phase cultures of MG1363acmAΔ1 containing either pGKAL1, encoding AcmA, or pGKAL5, encoding the AcmA derivative lacking all repeats (A₁₋₂₁₈). Right panel, zymographic analysis of AcmA activity in a renaturing SDS-10% polyacrylamide gel containing 0.15% *M. lysodeikticus* auto-claved cells of the same samples and the same amount of sample used in the left panel.

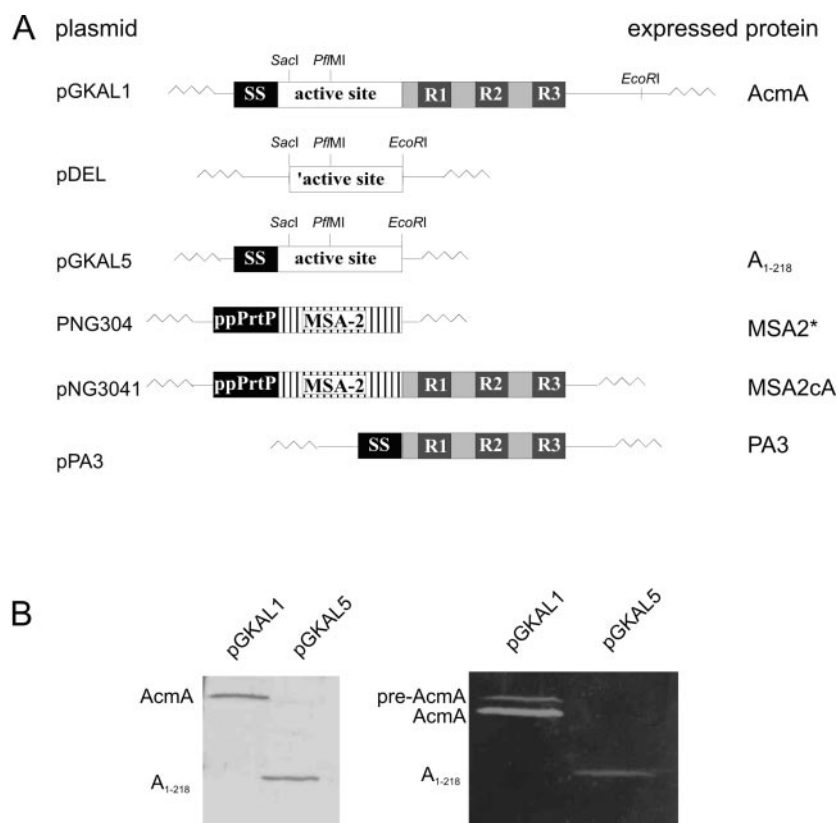


TABLE II

Properties of *L. lactis* MG1363acmAΔ1 expressing AcmA derivativesThe results obtained for the Reduction in A₆₀₀, PepX activity and halo formation are the average of two independent experiments.

Plasmid in acmAΔ1	AcmA ^a	Reduction in A ₆₀₀ ^b	PepX activity ^c	Halo ^d	Sedimentation ^e	AcmA activity ^f		Cell binding ^g
						sup	cfe	
pGK13	—	15.2	0.3	0	+	—	—	—
pGKAL1	+	36.7	19.8	5.0	—	+	+	+
pGKAL5	A ₁₋₂₁₈	15.6	0.3	0	+	+	+	—

^a A₁₋₂₁₈: AcmA lacking all three LysM repeats.^b The A₆₀₀ reduction was calculated using the formula, [(A_{max} - A_{60 h})/A_{max}] × 100%.^c Activity is in arbitrary units measured as the increase of absorption at 405 nm in time.^d The sizes of the halos were measured in millimeters from the border of the colony after 45 h of incubation at 30 °C.^e Analyzed by visual inspection of standing G_{1/2}M17 cultures after overnight growth in test tubes.^f In zymograms of samples from end-exponential phase G_{1/2}M17 cultures; sup, supernatant fraction; cfe, cell-free extract.^g Binding of AcmA derivatives in supernatants of end-exponential phase G_{1/2}M17 cultures to end-exponential phase cells of *L. lactis* MG1363acmAΔ1 after 20 min of incubation at 30 °C (see "Experimental Procedures" for details).

active than AcmA, but expression of both proteins is equal, as is shown using Western analysis with anti-AcmA antibodies (Fig. 1B). In the supernatant of cultures producing full-length AcmA or A₁₋₂₁₈, lytic activities could be detected corresponding to the calculated molecular weights of the processed, secreted forms of the respective proteins (40.3 and 17.1 kDa) (results not shown). These data show that the N-terminal domain of AcmA is active *in vitro* and, thus, contains the active site of the enzyme. This truncated form of AcmA is not able to lyse cells or separate chains *in vitro*, suggesting that the C-terminal domain of AcmA is required for both of these processes.

The C-terminal Domain of AcmA Is Required for Cell Wall Binding and Can Target a Heterologous Protein to Lactococcal Cells—Proof that the C-terminal domain (cA) of AcmA is required for cell binding was obtained by mixing supernatant fractions of end-exponential phase cultures containing AcmA or A₁₋₂₁₈ with cells of *L. lactis* MG1363acmAΔ1. After incubation, cells and supernatant were separated and examined for the

presence of lytic activity using zymographic analysis. AcmA was mainly present in the cell fraction, whereas A₁₋₂₁₈ was still present in the supernatant, indicating that the latter had lost its cell binding abilities (results not shown). The breakdown products of AcmA were also capable of binding to the lactococcal cells (data not shown). As shown by Poquet *et al.* (4), these breakdown products of AcmA are generated by the housekeeping surface protease HtrA. To investigate whether cA is capable of targeting heterologous proteins to the cell surface of *L. lactis*, a fusion protein containing the human malaria parasite *Plasmodium falciparum* surface antigen MSA2 and cA was constructed. The entire fusion protein was produced by nisin induction using the pNZ8048 (32) derivative pNG3041 (Fig. 1A) in *L. lactis* NZ9000. The fusion consists of the 184-amino acid preprosequence of the lactococcal proteinase PrtP for proper secretion in *L. lactis*, followed by MSA2 (223 amino acid residues) and cA (Fig. 1A). MSA2cA is secreted as a 62.5-kDa protein. *L. lactis* NZ9000 (pNG304) expresses a fusion protein

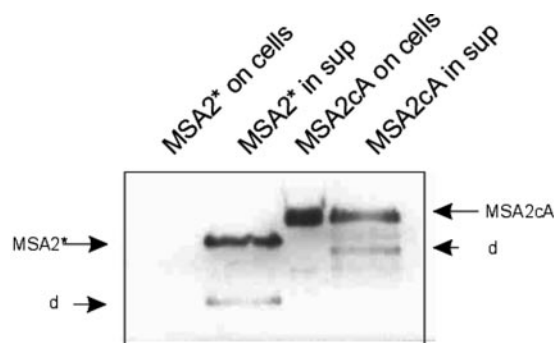


FIG. 2. Western blot analysis of binding of MSA2* and MSA2cA to whole lactococcal cells. Stationary phase cells from 1 ml of culture were mixed with the supernatant of induced cultures of *L. lactis* NZ9000 (pNG304), expressing MSA2*, and *L. lactis* NZ9000 (pNG3041), expressing MSA2cA. After allowing 5 min of binding, cells were collected by centrifugation. Proteins bound to cells and proteins in supernatant fractions were separated by SDS-10% PAGE and blotted onto polyvinylidene difluoride membranes. MSA2 antigen was visualized using MSA2-specific antisera and subsequent chemiluminescence detection. *d*, degradation products of MSA2* and MSA2cA.

containing the preprosequence of PrtP and the MSA2 protein, without cA (MSA2*).

The supernatants of cells secreting MSA2cA or MSA2* upon nisin induction were mixed with cells of *L. lactis* MG1363acmAΔ1. After incubation, cells and supernatants were separated and examined for the presence of MSA2 antigen by Western immunoblotting. As shown in Fig. 2, MSA2* was mainly present in the supernatant, whereas MSA2cA fractionated with the lactococcal cells, showing that cA is capable of specifically targeting MSA2 to the cell surface.

Acma and MSA2cA Bind to Other Gram-positive Bacteria—A whole-cell-binding assay was performed to examine whether Acma and MSA2cA are capable of binding to other Gram-positive bacteria. Washed cells of several bacterial species were mixed with Acma or MSA2cA. Protein binding was examined using zymographic analysis (Acma) and Western immunoblotting (MSA2cA). Acma and MSA2cA were both capable of binding to cells of *B. subtilis*, *E. faecalis*, *S. thermophilus*, *L. casei*, *L. sake*, *Lactobacillus buchneri*, *Lactobacillus plantarum*, *Listeria innocua*, and *Clostridium beijerinckii* (results not shown, but see below). The C-terminally truncated variant of Acma, A₁₋₂₁₈, and MSA2* did not bind to cells of these bacteria (data not shown).

Binding of cA to Cells of Gram-positive Bacteria Is Localized—After incubation with cells of *L. lactis*, *E. faecalis*, *S. thermophilus*, *L. casei*, *L. sake*, and *B. subtilis*, MSA2cA could be detected on the cell surface by immunofluorescence microscopy using anti-MSA2 antibodies and a fluorescent secondary antibody. MSA2cA was present at specific locations on the cell surface in all cases (Fig. 3A). MSA2cA clearly binds at the poles of *L. casei* cells. In the case of the cocci *L. lactis*, *E. faecalis*, and *S. thermophilus*, the protein binds around the septum. Binding to *B. subtilis* is at spots all over the cell surface, whereas MSA2cA binds to the entire cell surface of *L. sake* but not to the poles.

The cA Domain Binds to Peptidoglycan—Binding of cA to the cells of several different Gram-positive bacteria implies that the LysM repeats bind a cell wall component present in all of these cells. The localized binding observed above suggests either that this component is only present at specific loci or that it is present all over the surface but shielded at certain sites. To determine to which component cA binds, equal aliquots of lactococcal cell walls were treated with SDS to remove cell wall-associated proteins or with trichloroacetic acid, which is believed to remove peptidoglycan-associated polymers, among which are carbohydrates such as (lipo)teichoic acids (33).

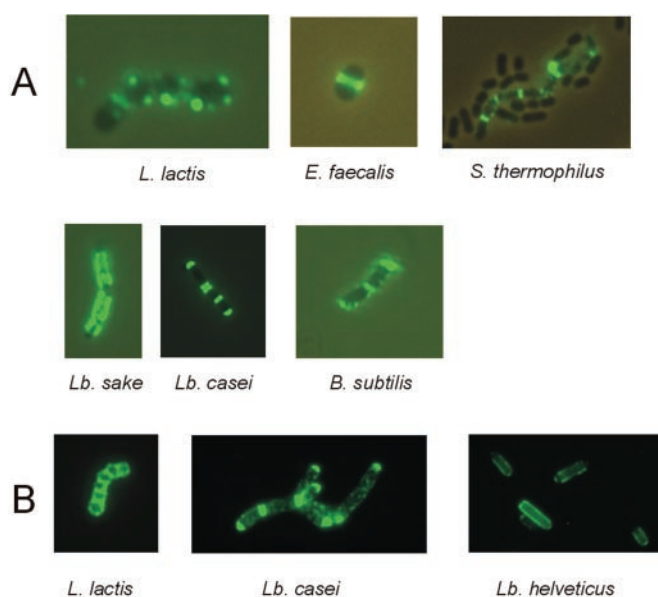


FIG. 3. A, localization of MSA2cA protein on several Gram-positive bacteria. Cells of overnight cultures of *L. lactis*, *E. faecalis*, *S. thermophilus*, *L. casei*, *L. sake*, and *B. subtilis* were mixed with supernatant of *L. lactis* NZ9000 (pNG3041) containing MSA2cA protein. Surface-bound protein was subsequently detected by immunofluorescence microscopy using anti-MSA2 rabbit polyclonal antibodies and anti-rabbit antibodies conjugated with the fluorescent probe Oregon Green (Molecular Probes, Inc., Eugene, OR). Bound MSA2cA protein is visible as bright green patches on the cell surfaces. B, localization of MSA2cA on chemically treated cells. Cells from *L. lactis* and *L. casei* were boiled for 15 min in 10% trichloroacetic acid. *L. helveticus* cells were boiled for 15 min in 10% SDS. The boiled and washed cells were mixed with MSA2cA-containing supernatants from *L. lactis* NZ9000 (pNG3041). MSA2cA protein was visualized using immunofluorescence as in A.

Treated cell walls were subsequently incubated with a supernatant containing MSA2cA, followed by Western analysis. Fig. 4 shows that MSA2cA binds to both SDS- and trichloroacetic acid-treated cell walls, which suggests that it binds to the peptidoglycan of the cell wall. Similar results were obtained with chemically treated cell walls from *B. subtilis* (Fig. 4).

Next, purified peptidoglycan from *S. aureus* was incubated with MSA2cA or MSA2* and pelleted by centrifugation. Fig. 5A shows that MSA2cA associates with peptidoglycan, whereas MSA2* is solely present in the supernatant. The bacteria mentioned above all have A-type peptidoglycan. To test whether MSA2cA was able to bind to B-type peptidoglycan, increasing amounts of MSA2cA (from supernatant) were added to 50 μ g of peptidoglycan isolated from *M. luteus* (a typical A-type peptidoglycan) or *C. flaccumfaciens* (B-type), and bound protein was analyzed by Western immunoblotting. *M. luteus* and *C. flaccumfaciens* were shown to bind similar amounts of MSA2cA. Breakdown products of the protein are also able to bind, as is visible in Fig. 5B.

MSA2cA binding on trichloroacetic acid-treated cells of *L. lactis* and *L. casei* was examined by immunofluorescence microscopy. Fig. 3B shows that MSA2cA is able to bind to the entire cell surface and not only to the poles of the trichloroacetic acid-treated cells. SDS treatment did not alter the localization of the bound protein (results not shown).

Binding of Acma or MSA2cA to *L. helveticus* is very inefficient (results not shown). The strain used for these experiments (*L. helveticus* ATCC 15009) is known to produce an S-layer (34). After boiling of the cells in SDS to remove the S-layer (35), binding of MSA2cA was more efficient and occurred over the whole *L. helveticus* cell surface (Fig. 3B). This result indicates that S-layer proteins hinder binding of MSA2cA.

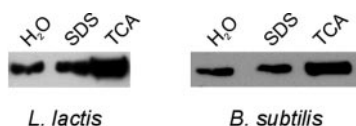


FIG. 4. Binding of MSA2cA to chemically treated cell walls of *L. lactis* and *B. subtilis*. Cell walls were isolated as described under "Experimental Procedures." Equal amounts of cell walls were boiled in water, 10% SDS, or 10% trichloroacetic acid and washed extensively with water. Subsequently, 1 ml of supernatant of an induced culture of *L. lactis* NZ9000 (pNG3041) containing MSA2cA protein was incubated for 5 min with the cell walls (0.5 mg). Subsequently, cell walls were subjected to centrifugation and were loaded onto an SDS-10% polyacrylamide gel. After electrophoresis, the MSA2 antigen was detected by Western immunoblotting using anti-MSA2 antibodies.

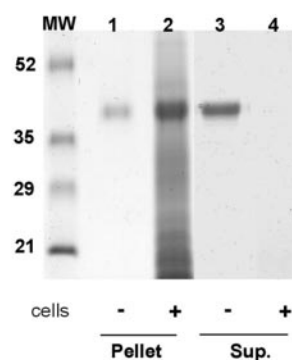


FIG. 6. Binding of purified PA3 protein to trichloroacetic acid-treated cells of *L. lactis*. PA3 was purified as described under "Experimental Procedures" and mixed with trichloroacetic acid-treated cells. After centrifugation, cell and supernatant fractions were analyzed for the presence of PA3. As a control, samples without cells were analyzed. A PA3 protein sample was centrifuged, and the pellet (PA3 aggregate; lane 1) and the supernatant (nonaggregated PA3; lane 3) were analyzed on a Coomassie-stained gel. The same amount of PA3 protein was mixed with trichloroacetic acid-treated cells, and after centrifugation the pellet (bound PA3; lane 2) and supernatant fraction (unbound PA3; lane 4) were analyzed on the same gel.

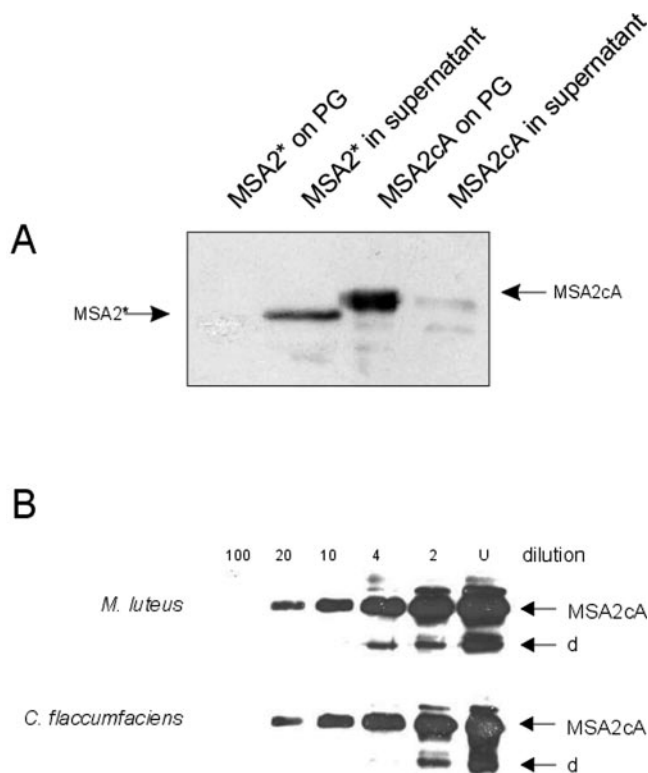


FIG. 5. A, MSA2cA binds to purified peptidoglycan from *S. aureus*. Purified peptidoglycan (0.5 mg) isolated from *S. aureus* (Fluka) was mixed with supernatants from nisin-induced cultures of *L. lactis* NZ9000 (pNG304), containing MSA2* protein, and *L. lactis* NZ9000 (pNG3041), containing MSA2cA. After 5 min of incubation, the peptidoglycan was subjected to centrifugation. Peptidoglycan and supernatant fractions were analyzed by Western immunoblotting using anti-MSA2 antibodies. PG, peptidoglycan (bound fraction); sup, supernatant after incubation with peptidoglycan (nonbound fraction). B, MSA2cA binds to A- and B-type peptidoglycan. MSA2cA protein was diluted 100, 20, 10, 4, or 2 times in MG1363acmAΔ1 supernatant. Also, undiluted protein (U) was used. The diluted protein was mixed with 50 µg of peptidoglycan isolated from *M. luteus* or *C. flaccumfaciens*, and bound protein was analyzed using Western immunoblotting.

For all of the above mentioned binding experiments, we used *L. lactis* supernatant containing the MSA2cA protein. *L. lactis* PA1001 (pPA3) contains a pNZ8048 derivative that expresses the cA domain (amino acids 220–437). Its secretion is driven by the signal sequence of the lactococcal USP45 protein (36) (see Fig. 1A for a diagram of PA3 protein). To exclude a role for an unidentified component present in the growth medium in binding to peptidoglycan, pure cA protein (PA3) was used in binding studies. PA3 was shown to fractionate with trichloroacetic acid-pretreated *L. lactis* cells, showing that the cA domain itself binds to peptidoglycan (Fig. 6, lane 2). No PA3 protein was found in the supernatant fraction (Fig. 6, lane 4). The smear in lane 2 of Fig. 6 is caused by the (partly) degraded proteins in

the trichloroacetic acid-pretreated cells.² When the same amount of PA3 was centrifuged without adding any trichloroacetic acid-pretreated cells, a small amount of PA3 protein was found in the pellet (Fig. 6, lane 1), probably representing aggregates of PA3. However, since similar PA3 samples were used in both experiments, all soluble proteins (Fig. 6, lane 3) specifically bound to trichloroacetic acid-pretreated cells (Fig. 6, compare lanes 3 and 4).

Lipoteichoic Acids Are Localized in *L. lactis* Cell Walls—*L. lactis* subsp. *cremoris* SK110 contains galactosyl residues in its lipoteichoic acids (LTA). Sijsma *et al.* (37) have shown that the galactosyl moiety obstructs the attachment of bacteriophage to its binding site in the cell wall. Strain SK112, a derivative of SK110 lacking galactosyl residues in its LTA, is phage-sensitive. The lectin RCA120, which is specific for terminal galactosyl units, binds specifically to the LTA isolated from SK110 and not to LTA from SK112 (37). Fluorescein-labeled RCA120 binds to those regions in the cell surface of SK110 that are not bound by MSA2cA (Fig. 7). Since galactosyl residues are only present in LTA (37), this would indicate that LTA in the cell wall of *L. lactis* SK110 is present only at the nonpolar sites. After boiling of the cells in 10% trichloroacetic acid, no galactosyl-containing polymers could be detected in the cell wall of SK110 using the RCA120 binding assay (results not shown), whereas MSA2cA binds to the entire surface of these trichloroacetic acid-treated SK110 cells. RCA120 did not bind to cells of MG1363.

DISCUSSION

In this paper, we show that AcmA is a modular enzyme consisting of an N-terminal active site domain and a C-terminal substrate-binding domain, containing three repeated LysM domains. A repeatless AcmA mutant was constructed and shown to be active on *M. lysodeikticus* cell walls, albeit with severely reduced efficiency. Cells expressing this derivative grew in long chains, sedimented, and did not autolyze. These results indicate that, although the N terminus of AcmA contains the active site, the presence of the C-terminal domain is needed for the enzyme to retain appreciable activity. It is tempting to speculate that this apparent increase in catalytic

² A. Steen, G. Buist, K. J. Leenhouts, M. El Khatibi, F. Grijpstra, A. L. Zomer, G. Venema, O. P. Kuipers, and J. Kok, unpublished observation.

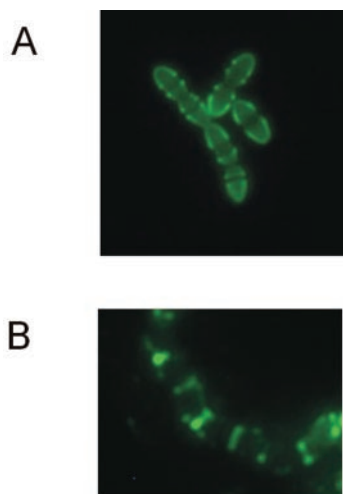


FIG. 7. A, localization in *L. lactis* SK110 cells of lipoteichoic acids substituted with galactosyl units. Cells were mixed with fluorescein-labeled RCA120 (10 μ g/ml), washed, and examined by fluorescence microscopy. B, MSA2cA binding to cells of *L. lactis* SK110 as visualized by immunofluorescence microscopy.

efficiency of AcmA is caused by the repeat domain by allowing the enzyme to bind to its substrate, the peptidoglycan of the cell wall. As was postulated by Knowles *et al.* (38) for the cellulase binding domains in cellobiohydrolases, such binding would increase the local concentration of the enzyme. The repeats could be involved in binding alone or could be important for proper positioning of the catalytic domain toward its substrate.

The C-terminal domain cA consists of three LysM domains separated by nonhomologous sequences. The hypothesis that the LysM domains of AcmA are involved in cell binding (2) was corroborated in this study. First of all, we show that AcmA is indeed capable of binding to bacterial cells. To prove that it was the C terminus of AcmA that facilitated binding and not some intrinsic cell wall binding capacity of the N-terminal domain, both the active site domain and the cA were separately produced in *L. lactis*. The active site domain A₁₋₂₁₈ did not bind to lactococcal cells, whereas the cA domain did bind to these cells when added from the outside. Cell wall binding was also obtained for the fusion protein MSA2cA in which the repeat domain was fused to the human malaria parasite antigen MSA2*, whereas MSA2* did not bind to lactococcal cells. In a previous study, Buist *et al.* (1) have shown that AcmA can operate intercellularly; AcmA-free lactococcal cells can be lysed when grown together with cells producing AcmA. Combining this observation with the results presented above allows us to conclude that AcmA does not only bind when confronting a cell from the outside but, indeed, is capable of hydrolyzing the cell wall with concomitant lysis of the cell.

LysM domains are present in proteins of many different bacteria and also of eukaryotes (10). The presence of similar repeats in proteins of different bacterial species strongly suggests that they recognize and bind to a cell wall component that is common to these bacteria. We show here that the LysM domains of AcmA bind specifically to peptidoglycan, the major cell wall component in bacteria. Binding is observed to many different bacteria and to different peptidoglycan types, suggesting that the LysM domains recognize a part common in all peptidoglycans. The peptidoglycans used in this paper have different structures; *L. lactis*, *S. aureus*, *B. subtilis*, and *M. luteus* peptidoglycans belong to the A-type peptidoglycan. Although there are differences in the composition of the peptide part, the A-type peptidoglycans all have L-Ala as the first amino acid, which attaches the peptide to the glycan chain.

Besides that, the cross-link in this type of peptidoglycan is always between the fourth (D-Ala) and the third amino acid (always a diaminoacid, such as *meso*-diaminopimelic acid (*e.g.* *B. subtilis*) or L-Lys (*e.g.* *S. aureus*)) (39). *C. flaccumfaciens* peptidoglycan is a B-type peptidoglycan; the first amino acid residue is not L-Ala but L-Gly. Cross-linking of the peptidoglycan is achieved by formation of a bond between D-Ala in the fourth position and the second amino acid (D-Glu) in the other peptide chain (39). The only moiety that A-type and B-type peptidoglycans have in common is the *N*-acetylglucosamine-*N*-acetylmureine polymer (glycan) (39). Furthermore, these structural differences between the peptidoglycans tested in this study, like amino acid composition and mode of cross-linking, did not seem to influence the efficiency of binding (Fig. 5B). Hence, we postulate that the LysM domain binds to glycan. The exact binding site in peptidoglycan is currently under study.

Although peptidoglycan is present on the entire cell surface of Gram-positive bacteria, the MSA2cA protein was not able to bind to the whole cell surface of *L. lactis*, *L. casei*, *L. sake*, and *B. subtilis*, as was shown by immunofluorescence microscopy studies. Apparently, other cell wall constituents prevent binding of a protein containing LysM domains, allowing binding only to those sites on the cell surface where this component is absent. These notions of localized binding fit with a very early observation that lysis in *L. lactis* starts at a specific locus, the equatorial ring (40). The sensitivity of the assay does not allow us to exclude the possibility that low amounts of protein can bind to the whole surface of the lactococcal cell, but the acid treatment clearly shows that the removal of cell wall components increases the binding capacity of the cell surface significantly. When AcmA-specific antibodies were used in immunofluorescence microscopy studies, no signal could be detected on the cell surface of *L. lactis* cells expressing AcmA. Expression of AcmA is probably too low to perform such a study (1).

The *S. aureus* autolysin Atl is specifically targeted to the cell division site by a repeat domain (41). The three repeats in Atl are not homologous to LysM domains, but they serve the same function in directing the enzyme to its site of action. Baba *et al.* (41) conclude that site-specific targeting of muralytic enzymes cannot be achieved by enzyme-substrate interactions, because the substrate peptidoglycan is present on the whole cell surface. In their view, localized binding could only be achieved by using a localized cell wall component. Here we show, however, that LysM domains do bind peptidoglycan. Mur of *L. citreum* and Mur1 of *S. thermophilus* are devoid of substrate binding domains and only contain an active site domain. Nevertheless, they have been shown to bind to the cell wall of the producing strains. A clear function of the cell wall binding domain of a peptidoglycan hydrolase is to direct the enzyme to its site of action, to the poles of the cell in the case of AcmA. Mur and Mur1 probably bind less specifically to the cell wall, probably even to the whole cell surface, which was already suggested for Mur (8).

HtrA is responsible for breakdown of AcmA (4) and also the fusion protein MSA2cA.³ The protease cleaves in cA (42), and the breakdown products still retain cell binding ability (Fig. 5B), showing that not all three LysM domains are necessary for binding.

The component(s) that prevent the LysM domains from binding all over the cell surface are removed by boiling cells or cell walls in trichloroacetic acid; after this treatment, the cell walls bind more MSA2cA (Fig. 4), and binding takes place over the entire cell surface (Fig. 3B). Trichloroacetic acid is believed to

³ A. Steen, G. Buist, K. J. Leenhouts, M. El Khatibi, F. Grijpstra, A. L. Zomer, G. Venema, O. P. Kuipers, and J. Kok, unpublished results.

remove cell wall-associated molecules (e.g. teichoic acids and polysaccharides) from the cell wall (33). The highly localized binding of AcmA also strongly suggests that the component hindering the binding of the enzyme is not present everywhere on the lactococcal cell surface. Chemical treatment of cell walls, especially extraction with trichloroacetic acid, has been used to identify bacteriophage receptors in the lactococcal cell wall. Extraction with trichloroacetic acid of cell walls from *L. lactis* strains F7/2, Wg2-1, and E8 resulted in a strong reduction of phage binding (33, 43). The reduction of phage binding correlated with the release of significant amounts of carbohydrates and phosphorus-containing material. The hypothesis that a carbohydrate cell wall component covalently linked to peptidoglycan was the phage receptor in *L. lactis* E8 was confirmed by lectin binding studies. When lectins that specifically bind galactose or glucosamine were mixed with cells of this strain, they prevented binding of phages. This revealed that galactose and glucosamine were required for the adsorption of the phages.

L. lactis SK110 contains LTA with galactosyl units and is resistant to phage attack (37). Using the fluorescein-labeled lectin RCA120, we show that galactosyl-decorated LTA is not uniformly distributed over the cell wall of strain SK110 and seems to be present at those sites to which MSA2cA does not bind; whereas MSA2cA mainly binds to the poles and the septum, RCA120 binds to the entire cell except the poles and septum. Assuming that all LTA is decorated with galactosyl residues in this strain, this would suggest that LTA is present at specific sites on *L. lactis* cells and that LTA is involved in the hindering of binding of MSA2cA to the cell surface. The galactosyl moiety of LTA is not involved in this phenomenon, since no galactosyl residues could be detected on the cell surface of MG1363, whereas MSA2cA binds to MG1363 cells at the same specific loci as seen in SK110. LTA, a decoration other than galactosyl residues, or a cell wall carbohydrate associated with LTA present in both MG1363 and SK110 is probably involved in hindering of LysM domain binding to peptidoglycan. The exact nature of this component is currently under study.

We have shown in this paper that *L. lactis* regulates the binding of AcmA by allowing the autolysin to bind only there where it is needed. In doing so, *L. lactis* protects itself against the potentially lethal activity of its autolysin.

Acknowledgments—We thank Anne de Jong for advice on and support of the computer work. We thank Prof. Dr. Ranjan Ramasamy for the anti-MSA2 antibodies. Peptidoglycans isolated from *C. flaccumfaciens* and *M. luteus* were a kind gift from Prof. S. J. Foster. We thank Aat Ledebouwer and Wouter Musters for discussions and suggestions.

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